The First-week Proliferative Response of Peripheral Blood PD-1\(^+\)CD8\(^+\) T Cells Predicts the Response to Anti-PD-1 Therapy in Solid Tumors

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**Abstract**

**Purpose:** To investigate blood-based dynamic biomarkers that predict responses to anti–programmed cell death protein 1 (PD-1) therapy in solid tumors.

**Experimental Design:** Preplanned biomarker analysis was performed as part of a phase II clinical trial (NCT02607631) in patients with metastatic or refractory thymic epithelial tumors (TETs; \(n = 31\)) who received pembrolizumab. The biomarker was further tested in an independent cohort of prospectively recruited patients with metastatic non–small cell lung cancer (NSCLC) who received pembrolizumab or nivolumab (NSCLC cohort 1; \(n = 33\)) and validated in an independent cohort of patients with NSCLC (NSCLC cohort 2; \(n = 46\)). Peripheral blood samples were obtained immediately before treatment (D0) and 7 days after the first dose (D7) and analyzed using multi-color flow cytometry.

**Results:** A higher fold-change in the percentage of Ki-67\(^+\) cells among PD-1\(^+\)CD8\(^+\) T cells 7 days after the first dose (Ki-67\(_{D7/D0}\)) significantly predicted durable clinical benefit (DCB; \(P < 0.001\)) and prolonged progression-free survival (PFS; \(P = 0.027\)) in patients with TETs. Ki-67\(_{D7/D0}\) \(\geq 2.8\) was also associated with better DCB, PFS, and overall survival (OS) in NSCLC cohort 1 (all \(P < 0.05\)). Ki-67\(_{D7/D0}\) was subsequently validated in NSCLC cohort 2, and Ki-67\(_{D7/D0}\) \(\geq 2.8\) significantly predicted better DCB (\(P = 0.001\)), PFS (\(P = 0.002\)), and OS (\(P = 0.037\)). Ki-67\(_{D7/D0}\) had a low correlation with tumor PD-L1 expression and combining both factors did not improve the predictive power of Ki-67\(_{D7/D0}\).

**Conclusions:** The proliferative response of peripheral blood PD-1\(^+\)CD8\(^+\) T cells, measured as the fold-change in the percentage of Ki-67\(^+\) cells 7 days after treatment (Ki-67\(_{D7/D0}\)), may be a useful surrogate biomarker for predicting the response and prognosis to anti-PD-1 therapy in solid tumors.

**Introduction**

Antibody-based therapies that block programmed cell death protein 1 (PD-1), such as pembrolizumab and nivolumab, exert their antitumor activity by reinvigorating exhausted CD8\(^+\) cytotoxic T cells (1, 2). A number of clinical trials have demonstrated the therapeutic efficacy of anti-PD-1 therapies against various human cancers, including malignant melanoma and non–small cell lung cancer (NSCLC; refs. 3–11). Because the clinical benefit of anti-PD-1 therapy is limited to a small proportion of patients, a great deal of effort has been made to identify biomarkers that predict the treatment response to this therapy (12, 13).

In clinical settings, programmed death-ligand 1 (PD-L1) expression in tumor tissues is widely used to select patient subgroups that have a high chance of responding to treatment (7, 8, 14). However, some trials have demonstrated that PD-L1 expression has an unsatisfactory power of prediction (5, 15). For example, in a phase III trial of patients with squamous cell NSCLC, the treatment response and survival rates were independent of PD-L1 expression (5). Other biomarkers, including tumor mutation burden (16), mismatch repair deficiency (17), immune microenvironment (18, 19), RNA signatures in tumor tissues (20), combinations of multiple clinicopathologic factors (21), and baseline monocyte frequency (22), have also been proposed as biomarkers for predicting the responsiveness to anti-PD-1 therapy, but none have shown sufficient predictive value for routine clinical use.

Dynamic biomarkers that can be evaluated during early treatment were recently suggested to have superior predictive power compared to static biomarkers that are measured at baseline (23). Dynamic biomarkers have been studied in serially acquired tumor tissue biopsies or peripheral blood samples from patients treated with anti-PD-1 (24–27). Although tumor tissue better reflects...
dynamic biomarkers in peripheral blood as a biomarker predicting treatment response, but it showed limited predictive ability (24, 25). One study found that the percentage of Ki-67⁺ cells among PD-1⁺CD8⁺ T cells 3 weeks after the first dose of pembrolizumab correlated with the tumor response in patients with melanoma, but only in combination with the initial tumor burden (24). Another study reported that CD8⁺ T cells in the peripheral blood of patients with NSCLC proliferated early after anti-PD-1 therapy, but that study did not demonstrate predictive ability (25).

In this study, we measured the percentage of Ki-67⁺ cells among peripheral blood PD-1⁺CD8⁺ T cells in samples collected before treatment and 7 days after the first dose, when the proliferative response of PD-1⁺CD8⁺ T cells peaked. We tested whether the fold-change in the percentage of Ki-67⁺ cells after the first week of treatment could predict treatment outcomes in 3 independent cohorts of patients with thymic epithelial tumors (TETs) and NSCLC. In addition, we examined factors that correlate with the proliferative response of PD-1⁺CD8⁺ T cells.

**Materials and Methods**

**Patients and sample collection**

Patients with histologically confirmed thymoma or thymic carcinoma who were not amenable to curative treatment and had progressed after at least 1 line of platinum-based chemotherapy were enrolled in a phase II trial (NCT02607631; n = 33). The aim of the trial was to evaluate the safety and efficacy of pembrolizumab (200 mg every 3 weeks). Patients were enrolled from March 2016 through June 2016 at Samsung Medical Center (Seoul, Republic of Korea; ref. 28). Peripheral blood was collected from 31 patients before treatment (day 0) and 7 days after they received the first dose of pembrolizumab. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using standard Ficoll-Paque (GE Healthcare) density gradient centrifugation. We also prospectively recruited 2 independent cohorts of patients with metastatic NSCLC who received pembrolizumab (200 mg every 3 weeks) or nivolumab (2 mg/kg every 2 weeks). NSCLC cohort 1 included patients enrolled between April 2016 and August 2017 at Samsung Medical Center (n = 33). NSCLC cohort 2 included patients enrolled when the Korean National Health Insurance Program started to cover the use of pembrolizumab and nivolumab in NSCLC patients, from September 2017 to April 2018 at Ulsan University Hospital (Ulsan, Republic of Korea; n = 11) and at Samsung Medical Center (n = 35). Peripheral blood was also collected from these patients on days 0 and 7 and the PBMCs were isolated. The biomarker discovered in the TET cohort was confirmed in NSCLC cohort 1 and subsequently validated in NSCLC cohort 2. On days 0, 7, and 21, we collected the peripheral blood from 9 patients with NSCLC to monitor the kinetics of immunologic responses. This study was approved by the Institutional Review Board of Samsung Medical Center and Ulsan University Hospital and was conducted in accordance with the Declaration of Helsinki. All patients provided informed consent before inclusion in the study.

**Evaluation**

Physical examination and laboratory tests were performed every 2 or 3 weeks. The tumor response was assessed by CT or MRI every 8 or 9 weeks according to the Response Evaluation Criteria In Solid Tumors (RECIST), version 1.1. Patients with TETs and NSCLC were followed with the same schedule. Durable clinical benefit (DCB) was defined as a complete response (CR), partial response (PR), or stable disease (SD) lasting longer than 6 months. Progression-free survival (PFS) was defined as the time from the start of anti-PD-1 therapy to either disease progression (according to RECIST v1.1) or death from any cause. Overall survival (OS) was defined as the time from the start of anti-PD-1 therapy to death from any cause.

**Multi-color flow cytometry**

Multi-color flow cytometry of PBMCs was performed at Korea Advanced Institute of Science and Technology (Daejeon, Republic of Korea), with the investigators blinded to the clinical outcome of the patients. The following fluorochrome-conjugated mAbs were used in multi-color flow cytometry: anti-CD8 (SK1 and RPA-T8), anti-CD3 (HIT3a), anti-CD4 (SK3), and anti-CD28 (CD28-2) from BD Biosciences; anti-CD1 (EH-12-2H7) and anti-Ki-67 (Ki-67) from BioLegend; anti-CD14 (61D3) and anti-CD19 (HIB19) from eBioscience; and anti-human IgG4 Fc (HP6025) from Southern Biotech. The therapeutic binding of pembrolizumab or nivolumab (human IgG4) to PD-1⁺ cells interfered with anti-Ki-67 staining in addition to anti-PD-1 staining to define PD-1⁻ cells. Dead cells were identified using the LIVE/DEAD Fixable Red Dead Cell Stain Kit (Invitrogen). Intracellular staining of Ki-67 was performed using a FoxP3 transcription factor staining buffer set (eBioscience) and specific antibodies. Tumor antigen–specific CD8⁺ T cells were detected by a PE-
conjugated MHC-I dextramer (HLA-A*02:01, NY-ESO-1-157; Immunex). HCMV-specific CD8+ T cells were detected by PE-conjugated MHC-I pentamer (HLA-A*02:01, pp65, Proimmune). All stained samples were acquired with an LSR II flow cytometer (BD Biosciences), and the data were analyzed by FlowJo software version 10.4.0 (Treestar). The gating strategies are summarized in Supplementary Fig. S1.

### PD-L1 IHC assay

PD-L1 expression was assessed at baseline using the PD-L1 IHC 22C3 pharmDx Assay Kit (Dako North America) in formalin-fixed, paraffin-embedded tumor tissue obtained before initiation of anti-PD-1 therapy (14). The percentage of tumor cells with membranous PD-L1 expression [tumor proportion score (TPS)] were evaluated. PD-L1 expression was classified as “high” if at least 50% of the tumor cells stained positive and “low” if 0%–49% of the tumor cells stained positive.

### Statistical analysis

Categorical variables and continuous variables were compared as indicated in the figure legends. The optimal cutoff point was determined as the point at which the Youden index was maximized by ROC curve. To assess the predictive power of composite biomarkers, ROC curves were generated using the predictive score of each individual calculated from a logistic regression model. AUIC values for paired ROC curves were compared with DeLong test using pROC package (version 1.13.0). Survival curves were generated using the Kaplan–Meier method and comparisons made using the log-rank test. To assess whether biomarkers had an independent significant effect on survival, we performed a multivariate Cox regression analysis that was adjusted for age, sex, histology, prior chemotherapy history, and tumor burden. Survival analysis was performed using the survival package (version 2.42-6). Regarding sample size, the TET cohort had 94% power to detect an effect size of 0.63, NSCLC cohort 1 had 86% power to detect an effect size of 0.53, and NSCLC cohort 2 had 89% power to detect an effect size of 0.47 all at the 5% significance level. The effect sizes were estimated from the rate of DCR in each cohort. Power calculations and effect size estimations were performed using the pwr package (version 1.2-2). Two-sided P values <0.05 were considered significant. All statistical analyses were performed with Prism software version 6.0 (GraphPad) and R statistical software (version 3.2.2, The R Foundation for Statistical Computing).

### Results

#### Patient characteristics

The baseline characteristics of the patients are summarized in Table 1. Patients with TETs (n = 31) received a median of 2 lines of previous systemic chemotherapy before pembrolizumab treatment and had no previous treatment with immune checkpoint inhibitors. Patients received a median of 9 cycles (range, 1–35 cycles) of pembrolizumab treatment. A best response of PR was observed in 6 patients (19.4%), SD in 18 patients (58.0%), and PD in 7 patients (22.6%). Eleven patients (35.4%) had DCR. The median follow-up time was 27.5 months (range, 25.3–28.9 months).

Patients in NSCLC cohort 1 (n = 33) received a median of 2 lines of previous systemic chemotherapy before pembrolizumab or nivolumab treatment and had no previous treatment with immune checkpoint inhibitors. Of the 33 patients, 16 received pembrolizumab and 17 received nivolumab. A median of 4 cycles...
(range, 1–21 cycles) and a median of 4 cycles (range, 2–53 cycles) were administered in patients who received pembrolizumab and nivolumab, respectively. A best response of PR was observed in 9 patients (27.3%), SD in 9 patients (27.3%), and PD in 15 patients (45.4%). Twelve patients (36.4%) had DCB. The median follow-up time was 18.5 months (range, 6.5–28.5 months).

In NSCLC cohort 2 (n = 46), the patients received a median 1 line of previous systemic chemotherapy and no immune checkpoint inhibitors. Pembrolizumab was administered in 38 patients and nivolumab was administered in 8 patients. A median of 6 cycles (range, 1–15) and a median of 9 cycles (range, 2–23) were administered in patients who received pembrolizumab and nivolumab, respectively. PR was observed in 11 patients (23.9%), SD in 19 patients (41.3%), and PD in 16 patients (34.8%). Nineteen patients (41.3%) had DCB. The median follow-up time was 10.9 months (range, 6.2–14.2 months).

Peripheral blood PD-1+CD8+ T cells show a proliferative response 1 week after anti-PD-1 therapy

A previous study examined the percentage of Ki-67+ proliferative cells among peripheral blood PD-1+CD8+ T cells, which were previously reported to be enriched for tumor antigen–specific T cells (29), 3 weeks after the first dose of pembrolizumab and reported that this value could predict treatment outcomes, but only when combined with the initial tumor burden (24). We hypothesized that the proliferation of CD8+ T cells peaks earlier than 3 weeks after administration of the first dose of anti-PD-1 antibodies. When we examined PBMCs from 9 patients with NSCLC, we found that the percentage of proliferative (Ki-67+) cells among PD-1+CD8+ T cells significantly increased in the first 7 days after the first dose and then significantly decreased in the following 2 weeks (3 weeks after the first dose; Fig. 1A), indicating that the proliferative response peaked at 7 days. On the basis of these data, we decided to evaluate the proliferative response of PD-1+CD8+ T cells at 7 days after the first dose and found that the percentage of Ki-67+ cells among peripheral blood PD-1+CD8+ T cells significantly increased during the first 7 days in both discovery cohorts, TET cohort and NSCLC cohort 1 (Fig. 1B and C).

To confirm whether a proliferative response was exhibited by tumor antigen–specific CD8+ T cells, we examined the percentage of Ki-67+ cells in the gate of tumor antigen–specific, peripheral blood CD8+ T cells from HLA-A*0201+ patients exhibiting NY-ESO-1157 restricted NY-ESO-1 1 mRNA upregulation in their tumor tissues. HLA-A*0201–restricted NY-ESO-1 dextramer (HLA-A*0201: NY-ESO-1157) was used to detect tumor antigen–specific CD8+ T cells, and HLA-A*0201–restricted HCMV pp65 pentamer (HLA-A*0201: pp6599) was used to detect non tumor antigen–specific CD8+ T cells as a control. We found that the percentages of Ki-67+ cells significantly increased in NY-ESO-1–specific CD8+ T cells (Fig. 1D and E) on day 7, but not in HCMV pp65–specific CD8+ T cells (Fig. 1F and G), in both the TET cohort and NSCLC cohort 1.

The first-week proliferative response of PD-1+CD8+ T cells predicts tumor responses to anti-PD-1 therapy and clinical outcomes in patients with TETs

The proliferative response after anti-PD-1 therapy was assessed by the fold-change in the percentage of Ki-67+ cells among PD-1+CD8+ T cells on day 7 (Ki-67D7/D0). In the cohort of patients with TETs, Ki-67D7/D0 was significantly higher in patients with DCB than in those with no durable benefit (NDB; Fig. 2A).

Furthermore, Ki-67D7/D0 had a high predictive value for DCB [AUC 0.86, 95% confidence interval (CI), 0.71–1.00; P = 0.001; Fig. 2B]. An optimal cut-off of 2.8 for Ki-67D7/D0 was determined from the ROC curve. The sensitivity and specificity of this cut-off were 90.9% and 75.0%, respectively. The probability of DCB was significantly higher in patients with Ki-67D7/D0 ≥ 2.8 than in patients with Ki-67D7/D0 < 2.8, although 33.3% of patients with Ki67D7/D0 ≥ 2.8 progressed within 6 months and 6.3% of patients with Ki67D7/D0 < 2.8 presented with DCB (Fig. 2C).

Among patients with Ki-67D7/D0 ≥ 2.8, 5 achieved PR, 9 had SD, and 1 had PD, whereas among patients with Ki-67D7/D0 < 2.8, 1 achieved PR, 9 had SD, and 6 had PD as best response (P = 0.052; Supplementary Table S1). Furthermore, patients with Ki-67D7/D0 ≥ 2.8 had a significantly prolonged PFS but not OS (Fig. 2D and E). The median PFS was 8.7 months (95% CI, 4.3–13.2 months) in patients with Ki-67D7/D0 ≥ 2.8 and 3.9 months (95% CI, 1.2–6.6 months) in those with Ki-67D7/D0 < 2.8 (P = 0.037; Fig. 2D). The median OS was 14.8 months (95% CI, 0.0–37.0 months) in patients with Ki-67D7/D0 ≥ 2.8 and 15.2 months (95% CI, 0.0–33.0 months) in those with Ki-67D7/D0 < 2.8 (P = 0.78; Fig. 2E). A multivariate analysis adjusted for other clinicopathologic factors revealed that Ki-67D7/D0 was independently and significantly associated with PFS (adjusted HR (aHR) 0.27; 95% CI, 0.09–0.82; P = 0.020), whereas Ki-67D7/D0 was not significantly associated with OS (Supplementary Table S2).

Ki-67D7/D0 predicts tumor response to anti-PD-1 therapy and clinical outcomes in patients with NSCLC

We then tested Ki-67D7/D0 as a predictive biomarker in a cohort of patients with NSCLC (NSCLC cohort 1, n = 33), which has a larger application of anti-PD-1 therapy. Similar to the TET cohort, Ki-67D7/D0 was significantly higher in patients with DCB than in those with NDB (Fig. 3A). Ki-67D7/D0 significantly predicted DCB (AUC 0.78, 95% CI, 0.61–0.94; P = 0.009; Fig. 3B) and the same optimal cut-off of 2.8 was determined from the ROC curve. The probability of DCB was significantly higher in patients with Ki-67D7/D0 ≥ 2.8 than in patients with Ki-67D7/D0 < 2.8, though 37.5% of patients with Ki67D7/D0 ≥ 2.8 presented DCB (Fig. 3C). Among patients with Ki-67D7/D0 ≥ 2.8, 7 achieved PR, 5 had SD, and 4 had PD, whereas among patients with Ki-67D7/D0 < 2.8, 2 achieved PR, 4 had SD, and 11 had PD as best response (P = 0.053; Supplementary Table S3). The median PFS was 6.0 months (95% CI, 3.3–8.7 months) for patients with Ki-67D7/D0 ≥ 2.8 and 1.4 months (95% CI, 1.0–1.8 months) for patients with Ki-67D7/D0 < 2.8 (P = 0.004; Fig. 3D). The median OS was 13.8 months (95% CI, 10.0–17.5 months) in patients with Ki-67D7/D0 ≥ 2.8 and 2.0 months (95% CI, 0.0–4.6 months) in patients with Ki-67D7/D0 < 2.8 (P = 0.001; Fig. 3E).

Next, we validated Ki-67D7/D0 as a predictive biomarker in an independent cohort of patients with NSCLC (NSCLC cohort 2, n = 46). The percentage of Ki-67+ cells among peripheral blood PD-1+CD8+ T cells also significantly increased during the first 7 days in NSCLC cohort 2 (Fig. 4A). In addition, Ki-67D7/D0 was significantly higher in patients with DCB than in those with NDB (Fig. 4B) and Ki-67D7/D0 significantly predicted DCB (AUC 0.81, 95% CI, 0.68–0.94; P < 0.001; Fig. 4C). Using the predefined cut-off of 2.8, we stratified the patients with Ki-67D7/D0 ≥ 2.8 and Ki-67D7/D0 < 2.8. The probability of DCB was significantly higher in patients with Ki-67D7/D0 ≥ 2.8 than in patients with Ki-67D7/D0 < 2.8.

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< 2.8 the patients (Fig. 4D). Among patients with Ki-67_D7/D0 ≥ 2.8, 10 achieved PR, 10 had SD, and 6 had PD, whereas among patients with Ki-67_D7/D0 < 2.8, 1 achieved PR, 9 had SD, and 10 had PD as best response (P = 0.022; Supplementary Table S4). The median PFS was 10.9 months (95% CI, 4.7–17.1 months) for patients with Ki-67_D7/D0 ≥ 2.8 and 2.1 months (95% CI, 2.0–2.3 months) for patients with Ki-67_D7/D0 < 2.8 (P = 0.002; Fig. 4E). The median OS was not reached in patients with Ki-67_D7/D0 ≥ 2.8 and 7.0 months (95% CI not evaluable) for patients with Ki-67_D7/D0 < 2.8 (P = 0.037; Fig. 4F).

Multivariate analysis of Ki-67_D7/D0 adjusted for other clinico-pathologic variables confirmed that Ki-67_D7/D0 independently
correlated with both PFS (aHR 0.34; 95% CI, 0.19–0.60; P < 0.001) and OS (aHR 0.34; 95% CI, 0.17–0.75; P = 0.007; Supplementary Table S5) in the combined cohort of patients with NSCLC. Performance status also independently correlated with OS (aHR 2.98; 95% CI, 1.36–10.12; P = 0.013), but was not significantly associated with best tumor response (P = 0.15) and DCB (P = 0.23), implying that it was a prognostic factor rather than a predictive biomarker. We further analyzed the predictability of Ki-67D7/D0 separately in patients with squamous cell carcinoma (n = 34) and patients with nonsquamous histology (n = 45). Ki67D7/D0 significantly correlated with DCB, PFS, and OS in both squamous and nonsquamous NSCLC (Supplementary Table S6).

Next, we investigated which subpopulation within PD-1+CD8+ T cells responded to anti-PD-1 therapy in patients with NSCLC. We focused on CD28+ cells because recent studies reported that CD28 is a major target of the PD-1–mediated inhibitory signal and that anti-PD-1–induced T-cell reinvigoration depends on CD28 (30, 31). When we analyzed Ki-67D7/D0 in the CD28+PD-1+CD8+ and CD28+PD-1–CD8+ T-cell populations separately, we found that Ki-67D7/D0 was significantly higher in the CD28+PD-1+CD8+ T-cell population than in the CD28+PD-1–CD8+ T-cell population (Fig. 4G).

Combination of Ki-67D7/D0 and PD-L1 TPS as predictive biomarkers

Having established that Ki-67D7/D0 may serve as a promising biomarker in patients treated with anti-PD-1 therapy, we investigated the association between PD-L1 TPS, the most commonly used biomarker in practice, and Ki-67D7/D0. PD-L1 TPS was evaluable in 25 (80.6%) and 68 (86.1%) patients in the TET cohort (n = 31) and the combined NSCLC cohort (n = 79), respectively (Supplementary Table S7). No significant difference in Ki-67D7/D0 was observed according to the level of PD-L1 TPS in patients with TETs and NSCLC (Fig. 5A and B).

Next, we assessed whether combining PD-L1 TPS with Ki-67D7/D0 improves the predictive power compared with Ki-67D7/D0 as a single biomarker. In the TET cohort, PD-L1 TPS did not significantly predict DCB (AUC 0.54, 95% CI, 0.31–0.77; P = 0.73) and had lower predictive power than Ki-67D7/D0 (AUC 0.89, 95% CI, 0.76–1.00; P = 0.002) and the combination of Ki-67D7/D0 and PD-L1 TPS (AUC 0.91,
95% CI, 0.80–1.00; \( P = 0.001 \); Fig. 5C). In addition, the combination of Ki-67D7/D0 and PD-L1 did not significantly improve the predictive power compared with Ki-67D7/D0 alone (\( P = 0.32 \); Fig. 5C). In the NSCLC cohort, PD-L1 significantly predicted DCB (AUC 0.64, 95% CI, 0.51–0.77; \( P = 0.049 \)), but had lower predictive power than Ki-67D7/D0 (AUC 0.81, 95% CI, 0.70–0.91; \( P < 0.001 \)) and combination of Ki-67D7/D0 and PD-L1 TPS (AUC 0.82, 95% CI, 0.72–0.93; \( P < 0.001 \); Fig. 5D). No significant difference was found between the AUC values using Ki-67D7/D0 as a single biomarker and using the combination of Ki-67D7/D0 and PD-L1 TPS (\( P = 0.57 \); Fig. 5D).

**Discussion**

Immune checkpoint inhibitors that block the PD-1/PD-L1 interaction have shown promising results in patients with advanced cancers (3–8). Although patients with a tumor response commonly experience long-term tumor control, only a small fraction of patients experience clinical benefit with these inhibitors. Unfortunately, there is currently no robust biomarker that predicts the treatment outcome in response to anti-PD-1 therapy. In this study, we identified and validated a blood-based biomarker that predicted the response to anti-PD-1 therapy in 3 independent cohorts of 2 distinct types of cancer, TETs and NSCLC. We found that the fold-change in the percentage of Ki-67\(^{+}\) cells among peripheral blood PD-1\(^{+}\)CD8\(^{+}\) T cells 7 days after the first dose, a marker we termed Ki-67D7/D0, predicted tumor response and survival.

Peripheral blood CD8\(^{+}\) T cells are composed of T cells specific to various antigens. In this study, we showed that anti-PD-1 therapy increases the expression of Ki-67 in tumor antigen–specific T cells, but not in irrelevant virus-specific T cells, using MHC I multimers. However, detecting tumor-specific CD8\(^{+}\) T cells using MHC I multimers is limited to only a few HLA types and tumor antigens. In a previous study, tumor-specific CD8\(^{+}\) T cells were identified among peripheral blood PD-1\(^{+}\)CD8\(^{+}\) T cells of patients with cancer, but not in PD-1\(^{+}\)CD8\(^{+}\) T cells (29). Thus, the proliferative response in PD-1\(^{+}\)CD8\(^{+}\) T cells in patients with cancer may reflect the proliferative response of tumor-specific CD8\(^{+}\) T cells, even without MHC I multimers.

Considering the mode of action of anti-PD-1 blocking antibodies, it stands to reason that PD-1\(^{+}\)CD8\(^{+}\) exhausted T cells must be reinvigorated and recover their functions for patients to achieve long-term tumor control and show improved survival, regardless...
the type of tumor. Indeed, the proliferative response of tumor-infiltrating CD8\textsuperscript{+} T cells after anti-PD-1 therapy in patients with melanoma has been shown to be associated with tumor response (18). In our study, the proliferative response of PD-1\textsuperscript{+}CD8\textsuperscript{+} T cells after anti-PD-1 therapy was important for the tumor response in 2 distinct types of tumors, TETs and NSCLC. Ki-67D7/D0 exhibited high predictive ability for NDB, and 89% of the patients with Ki-67D7/D0 < 2.8 progressed within 6 months. This indicates that tumor control may hardly be achieved without the reinvigoration of PD-1\textsuperscript{+}CD8\textsuperscript{+} T cells early after anti-PD-1 therapy. This finding also implies that Ki-67D7/D0 may be used to screen for patients who are least likely to respond to anti-PD-1 therapy early after treatment initiation. However, there is still room for improvement of this new biomarker due to the false values. Thirty-seven percent of patients with Ki67D7/D0/C21 ≥ 2.8 progressed within 6 months, whereas 11% of patients with Ki67D7/D0 < 2.8 presented DCB. Combining other biomarkers with Ki-67D7/D0 may improve the predictive ability of Ki-67D7/D0 in discriminating the responders and nonresponders. By identifying the nonresponders in advance, patients may have an

Figure 4. Validation of Ki-67D7/D0 in NSCLC cohort 2. A, Percentage of Ki-67\textsuperscript{+} cells among PD-1\textsuperscript{+}CD8\textsuperscript{+} T cells at day 0 and day 7 in NSCLC cohort 2 (n = 46). B, The Ki-67D7/D0 value was compared between patients with NDB (n = 27) and those with DCB (n = 19). The box plot represents the IQR with the horizontal line indicating the median. Whiskers extend to a maximum of 1.5 × IQR beyond the box. C, The ROC curve to predict DCB after treatment. D, Percentage of DCB in patients with Ki-67D7/D0 ≥ 2.8 and with Ki-67D7/D0 < 2.8. Kaplan-Meier curves of PFS (E) and OS (F) in patients with Ki-67D7/D0 ≥ 2.8 or Ki-67D7/D0 < 2.8. G, Ki-67D7/D0 in CD28\textsuperscript{−}PD-1\textsuperscript{+}CD8\textsuperscript{+} T cells and CD28\textsuperscript{+}PD-1\textsuperscript{+}CD8\textsuperscript{+} T cells in patients with NSCLC (n = 79). The box plot represents the IQR with the horizontal line indicating the median. Whiskers extend to a maximum of 1.5 × IQR beyond the box. Representative multi-color flow cytometry plots on the right show Ki-67 expression in the CD28\textsuperscript{−}PD-1\textsuperscript{+}CD8\textsuperscript{+} and CD28\textsuperscript{+}PD-1\textsuperscript{+}CD8\textsuperscript{+} T cell gates. Statistical comparisons were performed using paired t test (A, G), Student t test (B), Pearson χ\textsuperscript{2} test (D), or the log-rank test (E and F). **P < 0.01; ***P < 0.001; ****P < 0.0001.)

of the type of tumor. Indeed, the proliferative response of tumor-infiltrating CD8\textsuperscript{+} T cells after anti-PD-1 therapy in patients with melanoma has been shown to be associated with tumor response (18). In our study, the proliferative response of PD-1\textsuperscript{+}CD8\textsuperscript{+} T cells after anti-PD-1 therapy was important for the tumor response in 2 distinct types of tumors, TETs and NSCLC. Ki-67D7/D0 exhibited high predictive ability for NDB, and 89% of the patients with Ki-67D7/D0 < 2.8 progressed within 6 months. This indicates that tumor control may hardly be achieved without the reinvigoration of PD-1\textsuperscript{+}CD8\textsuperscript{+} T cells early after anti-PD-1 therapy. This finding also implies that Ki-67D7/D0 may be used to screen for patients who are least likely to respond to anti-PD-1 therapy early after treatment initiation. However, there is still room for improvement of this new biomarker due to the false values. Thirty-seven percent of patients with Ki67D7/D0 ≥ 2.8 progressed within 6 months, whereas 11% of patients with Ki67D7/D0 < 2.8 presented DCB. Combining other biomarkers with Ki-67D7/D0 may improve the predictive ability of Ki-67D7/D0 in discriminating the responders and nonresponders. By identifying the nonresponders in advance, patients may have an
tor (33, 34). Ki-67D7/D0 is a dynamic immunologic response to PD-L1 interaction.

PD-L1 expression and Ki-67D7/D0. Ki-67D7/D0 did not significantly predict treatment outcome. However, the addition of PD-L1 TPS to Ki-67D7/D0 was not able to further improve the predictive power. Nevertheless, due to the small number of patients included in the analyses, future studies are needed to confirm these findings.

Given that the mode-of-action of PD-1 blocking antibodies and PD-L1 blocking antibodies are similar, further investigation of the predictive power of Ki-67D7/D0 in patients treated with atezolizumab or durvalumab may be an interesting subject of research. In addition, combination therapy of anti-PD-1/PD-L1 with other treatment modalities, such as chemotherapy and chemoradiotherapy, are being actively investigated (35, 36). In these studies, the clinical benefit of anti-PD-1/PD-L1 therapy was observed regardless of PD-L1 status. Thus, new biomarkers are needed in these treatment settings and Ki-67D7/D0 may be a good candidate for a predictive biomarker.

In this study, Ki-67D7/D0 predicted treatment outcome in 2 distinct types of tumors, TETs and NSCLC. Considering the mode-of-action of PD-1 blocking antibodies, measuring the extent of PD-1+CD8+ T-cell proliferation may be applicable to broader types of cancer. However, Ki-67D7/D0 significantly predicted OS in patients with NSCLC, but not in the patients with TETs. This implies that the predictive power of Ki-67D7/D0 may vary according to the type of cancer or histology and should be confirmed in future studies.

This study has some limitations. The sample size was small, patients with different types of histology were included, and follow-up time was short, which may limit the statistical power of these findings. Further studies are needed to investigate therapies that can reactivate these tumors. We also found that CD28+PD-1+CD8+ T cells preferentially proliferated in response to anti-PD-1 therapy compared with CD28−PD-1−CD8− T cells, which is in line with a recent study reporting CD28 dependency of the effect of PD-1 blockade on exhausted PD-1+CD8+ T cells (30, 31). These data, and the analyses, future studies are needed to confirm these findings.

Figure 5.
Combination of Ki-67D7/D0 and PD-L1 TPS as predictive biomarkers. A, Ki-67D7/D0 was compared between patients with TETs with low PD-L1 TPS (<50%, n = 12) and high PD-L1 TPS (≥50%, n = 13). B, Ki-67D7/D0 was compared between patients with NSCLC with low PD-L1 TPS (<50%, n = 27) and high PD-L1 TPS (≥50%, n = 41). The box plot represents the IQR with the horizontal line indicating the median. Whiskers extend to a maximum of 1.5 × IQR beyond the box. The ROC curve of Ki-67D7/D0 (blue solid line), PD-L1 TPS (black solid line), and the combination of Ki-67D7/D0 and PD-L1 TPS (green solid line) to predict DCB in patients with TETs (C) and NSCLC (D). Statistical comparisons were performed using Student t test (A and B), or Delong test (C and D). ns, not significant.
and interpretation of our data. Thus, our results must be further validated in larger cohorts with longer follow-up. In addition, we only tested 2 types of cancer and further validation should be performed in other types of cancer that are approved for pembrolizumab or nivolumab treatment, such as malignant melanoma, head and neck squamous cell carcinoma, urothelial cancer, and hepatocellular carcinoma.

In conclusion, this preliminary study suggests an association of the fold-change in the percentage of Ki-67+ cells among PD-1+ CD8+ T cells 7 days after the first dose of anti-PD-1 antibodies (Ki-67D7/D0) with treatment outcomes in patients treated with anti-PD-1 therapy. Further prospective studies with more patients with various types of cancer will be needed to validate Ki-67D7/D0 as a useful biomarker in patients with solid tumors treated with anti-PD-1 therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: K.H. Kim, M.-J. Ahn, E.-C. Shin

References


Correction: The First-week Proliferative Response of Peripheral Blood PD-1⁺CD8⁺ T Cells Predicts the Response to Anti-PD-1 Therapy in Solid Tumors

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In the original version of this article (1), Fig. 3A contained incorrect flow cytometry plots. The figure has been corrected in the latest online HTML and PDF versions of the article. The authors regret the error.

Reference

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